AN ELECTRON MICROSCOPE STUDY OF THE EGG MEMBRANES OF MELANOPLUS DIFFERENTIALIS (THOMAS) [1, 2]

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Extensive research has been carried out on the origin and the physical and chemical properties of the membranes surrounding the egg of the grasshopper *Melanoplus differentialis* (Thomas). The results of Campbell (1929), Jahn (1935a, 1935b, 1936), and Cole and Jahn (1937) seem most helpful in understanding its physical and chemical nature, while Slifer (1932, 1937, 1938a, 1938b), and Slifer and King (1934) give a clear picture of the structural relations of the egg membranes during embryonic development. It is the purpose of this paper to present results obtained from a study of the egg membranes with the aid of the RCA Electron Microscope, Model EMU-2B.

The outer membrane of the grasshopper's egg, the chorion (about 20 μ thick), is secreted by the cells of the maternal ovariole epithelium which enlarge during yolk deposition. Investigators seem to disagree regarding the formation and continuity of the vitelline membrane which lies just inside the inner surface of the chorion (Slifer, 1937). Since this membrane appears to become fragmentary as soon as embryonic development begins, it was not studied with the electron microscope.

At the time of laying, the egg, which has broken away from the ovariole epithelium, passes down the oviduct and out of the ovipositor into a pod made up of from 10 to 150 eggs. During the development of the blastoderm and its differentiation into germ band and serosa, very little change occurs in the egg membranes. The serosa cells migrate peripherally and completely surround the yolk and germ band by the fifth day (at 25° C.). They appear just inside the chorion as large, flat cells, with dense elliptical nuclei. During the sixth day, the serosa cells secrete on their periphery a non-chitinous (Campbell, 1929) membrane called the yellow cuticle (Jahn, 1935a, 1935b, and 1936). It is usually complete by the beginning of the seventh day at 25° C. Jahn (1936) found this thin membrane ($< 1\ \mu$) to show a high degree of ionic impermeability, and it may be closely related chemically to the cuticulin of *Rhodnius prolixus* (Wigglesworth, 1933).

The serosa cells also secrete a white fibrous membrane differing structurally and chemically from the yellow cuticle, and lying just inside of it. This layer, which gave Campbell and Jahn a positive chitosan test, is the white cuticle. Slifer's (1937) microscopical examination showed it "to be composed of innumerable fine threads tangled closely together." The deposition of this layered membrane (about 20 μ thick) requires one week at 25° C. (Slifer, 1937). She concluded, "The yel-

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low layer confers a high degree of impermeability; while the white layer is responsible for a greatly increased toughness and resistance to mechanical injury."

As the embryo develops, the chorion, if allowed to dry, cracks into an irregular pattern. The yellow cuticle is broken during the hatching process, but the embryo apparently is not strong enough to break the tough fibers of the white cuticle. Just before hatching, the latter, or the major portion of it, is digested away, making possible the emergence of the nymph. Slifer (1937, 1938b) has submitted evidence that the enzyme which is responsible for this digestion is produced by the pleuropodia.

The investigation of these membranes with the electron microscope requires special techniques and much patience. Since the electron beam is capable of penetrating tissue only about 1 μ in thickness, clear photographs of structures can only be made if the thickness is kept below 0.5 μ. There are many ways proposed for the preparing of extremely thin sections. All methods thus far noted in the literature fall into two categories: *A*. High speed microtomes, or *B*. Variations in the mechanics of sectioning.

Among the high speed microtomes which have been used and recommended are the "Cyclone Microtome" of O'Brien and McKinley (1943) and the two models by Fullam and Gessler (1946). One of the most difficult problems of the high speed microtome seems to be the locating of the sections after they are cut.

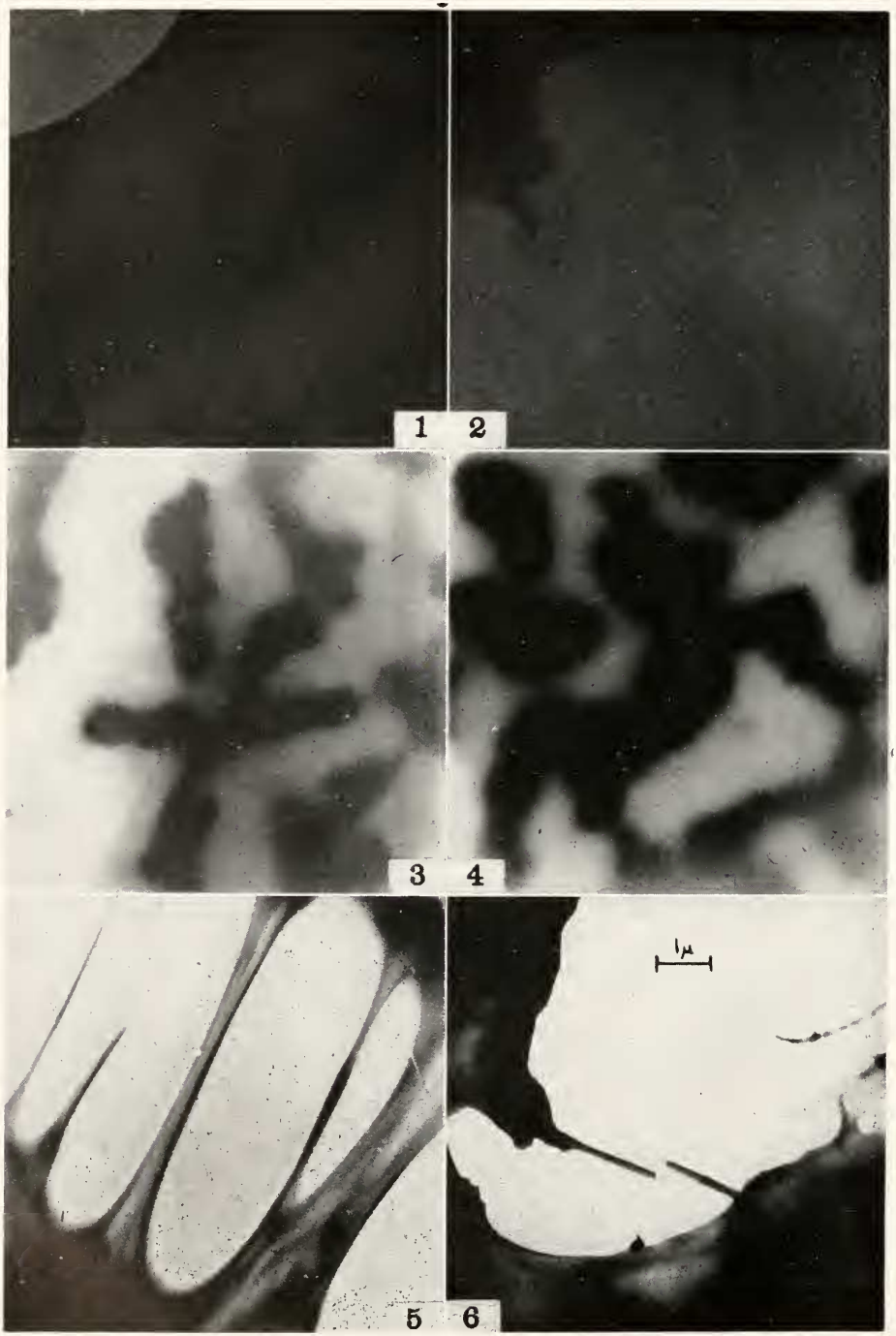
The earliest workers with the electron microscope tried many variations of geometry, mechanics, and chance. One of the methods, which involves a great deal of chance, is to cut the thinnest sections possible on the conventional microtome. These sections are re-embedded and re-sectioned, the operator hoping to obtain at least one very thin section out of many original cuts. The most commonly tried method, and the method used in this study, was the cutting of wedge-shaped sections on the conventional microtome as described by von Ardenne (1939), and Richards, Anderson and Hance (1942). From these a great many fine electronmicrographs have been produced. A very successful method used by Pease and Baker (1948) is the modification of the conventional Spencer Model 820 rotary microtome by decreasing the pitch of the diagonal backing plate to enable the cutting of 0.1 μ thick sections from doubly embedded material.

Another method of preparing sections, which has been used by Richards and his associates at the University of Minnesota, is to choose material which is naturally very thin. The results of these studies are reported by Richards and Anderson (1942), Anderson and Richards (1942), Richards and Korda (1947), and Richards and Korda (1948). The present investigator has employed this method in securing electronmicrographs of the yellow cuticle.

All membranes used were obtained from the eggs of animals kept under laboratory conditions as described by Boell (1935). To obtain the extremely thin sections of the membranes the following methods were used:

A. Fresh yellow cuticle. As stated above, the yellow cuticle is deposited by the serosa cells about the sixth day after the eggs are laid. Six-day-old eggs were placed in sodium hypochlorite solution to dissolve off the chorion (Slifer, 1945). The only membrane left enclosing the egg at this age is the yellow cuticle. The yellow cuticle of the egg was ruptured in isotonic saline solution, and a piece of it (after rinsing in isotonic saline solution and distilled water) is placed on the object screen of the electron microscope.

PLATE I



B. Preserved sections of chorion and white cuticle.

1. Geometrical method. One end of eggs at different stages of development was cut off, and the eggs fixed in Bouin and embedded in paraffin (56° MP) by the usual method. A number of eggs were embedded parallel in each paraffin block. The blocks were sectioned longitudinally or thereabouts in the conventional microtome at settings from 2–10 μ (Dempster, 1942). Some of the above eggs were prepared leaving yolk and embryo in the membranes, and some were prepared with the yolk and embryo removed before fixing. Little difference was noted in the results. The only sections having wedge characteristics were those cut from the eggs at the beginning and ending of the sectioning. These sections were placed in xylol to dissolve out the paraffin before being mounted on the object screen of the electron microscope.
2. Modifying Spencer Model 820 Rotary Microtome. With this modification, eggs were doubly embedded as described by Pease and Baker (1948).

The placing of the sectioned material upon the object screen or grid near the center is not without problems. Since the grid wires are opaque, they always obscure a part of the material from view.

Since the limit of adjustment of the holder is only about five meshes of the grid in diameter, the exact centering of the specimen is critical. In sections carefully prepared and mounted, it may turn out that the material to be observed will have a location behind a wire of the grid. The super-drying of the specimen in the vacuum chamber, plus the "baking" it receives from the electron beams, renders the material so fragile and brittle that its relocation on the grid is next to impossible.

Results

An examination of the electronmicrographs with their titles and explanations reveals the structure of the membranes of the grasshopper egg. These figures are presented from the many pictures taken, as typical of the materials examined. In general, as the eggs become older, the chorion and yellow cuticle become electronically more dense. Thinner sections of older membranes were necessary before the material could be viewed or the electronmicrographs taken.

Figures 1 and 2 of the chorion (using wedge-shaped preserved sections) indicate that it appears to have no clearly resolvable internal structure. The shades of gray of the electronmicrographs vary greatly with the thickness of the sections. Figure 1 is a rather thick section of chorion, while Figure 2 shows a thinner section, and the additional thickness of torn yellow cuticle is at the edge.

Plate I

Figure 1. At edge of chorion from 14 days postdiapause eggs. Preserved specimen. × 13,000.

Figure 2. Chorion from 14 days postdiapause eggs. Torn edge of white cuticle at top. Preserved specimen. × 13,000.

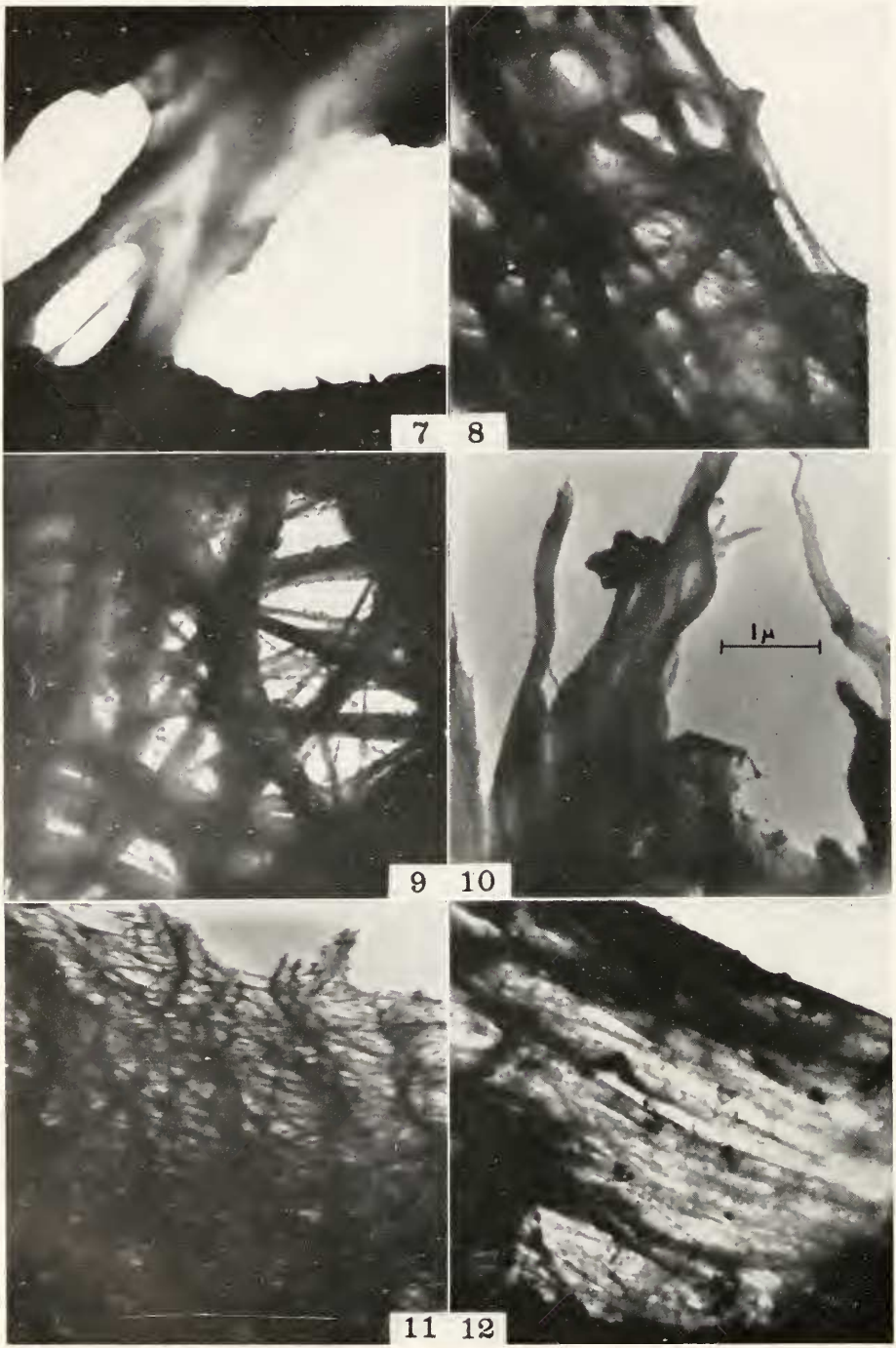
Figure 3. Yellow cuticle from 6-day-old eggs. Fresh specimen. × 13,000.

Figure 4. Same as Figure 3.

Figure 5. Stretched yellow cuticle from 6-day-old eggs. Fresh specimen. × 13,000.

Figure 6. Stretched yellow cuticle from 16 days postdiapause eggs. Preserved specimen. × 13,000.

PLATE II



Since wedge-shaped sections were used, no exact measurements of thickness were possible. All estimates or comparisons of thickness were the result of noting the distance from the thin edge of the wedge. Cross sections of preserved chorion, when stained with Delafield's hematoxylin and eosin or with Mallory's triple stain, appear under the high power oil immersion lens of the light compound microscope to be composed internally of short uneven fibers (Slifer, 1937, 1938a). However, the unstained, longitudinal, wedge-shaped sections of the chorion examined in the electron microscope seem to reveal no indications of a fiber-like structure. (See Figs. 1 and 2.)

Figures 3 and 4 of the yellow cuticle give evidence of the "minute ridges and tubercles" as described by Slifer (1937). These appear on the outer surface of this membrane. The ridges give greater thickness to the yellow cuticle and are believed to be responsible for the "Dalmatian-dog" pattern, which appears to be larger in the fresh material (Figs. 3 and 4) than in the preserved specimens (Figs. 13 and 14). This difference in size may be due to variations in material and shrinkage of the preserved material.

Figure 5 shows the results of stretching the fresh yellow cuticle which occurs as it dries in the electron microscope. Even in these stretched strands, variations in thickness are apparent. Figures 5 and 6 show preserved yellow cuticle which has been pulled to the point of breaking. Figures 5 and 6 both seem to indicate that there is stretching before the strands break. Note the blunt ends of the broken strands.

Figures 8 through 12 show the fibrous layered structure of the white cuticle. It was discovered that if the wedge-shaped sections were stained in eosin before being mounted on the grid in the electron microscope, the fibrous structure was largely obliterated. Since this was interpreted as an artifact, all stains were omitted on membranes employed in this study.

Decided differences of structure between the yellow and white cuticles, as evidenced in this and previous studies, point to the serosa cells as embryonic in nature and differing biochemically during development. The ability of the serosa cells to secrete two different structures or membranes, the yellow and the white cuticle within the same egg, indicates a similarity of function to the epidermal cells of insects (Wigglesworth, 1948).

Figures 8 and 9 show an extreme variation in the size of the fibers. There are indications of individual fibers and bundles of fibers both being present in the same white cuticle. No explanation is offered for the nodules on the fibers which are

PLATE II

FIGURE 7. Stretched yellow cuticle appearing at crack in chorion from 16 days postdiapause eggs. Preserved specimen. × 13,000.

FIGURE 8. White cuticle fibers from 11 days postdiapause eggs. Preserved specimen. × 13,000.

FIGURE 9. White cuticle fibers from 11 days postdiapause eggs. Preserved specimen. × 13,000.

FIGURE 10. Stretched white cuticle fibers from 11 days postdiapause eggs. Preserved specimen. × 22,200.

FIGURE 11. White cuticle fibers from 11 days postdiapause eggs. Preserved specimen. × 13,000.

FIGURE 12. White cuticle fibers from 11 days postdiapause eggs. Preserved specimen. × 13,000.

PLATE III

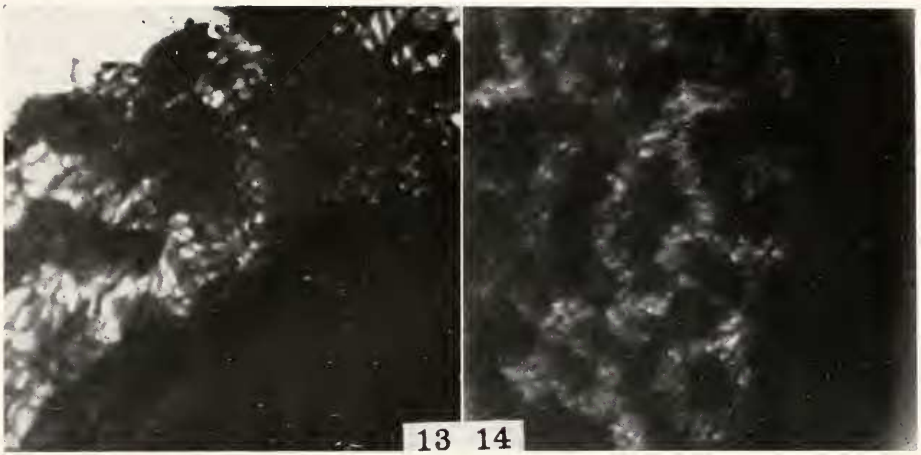


FIGURE 13. Yellow and white cuticle from 11 days postdiapause eggs. Preserved specimen. × 13,000.

FIGURE 14. Yellow and white cuticle from 11 days postdiapause eggs. Preserved specimen. × 13,000.

especially noticeable in Figure 9. Figure 10 shows the results of stretching white cuticle.

Figures 13 and 14 are combinations of yellow and white cuticle taken near the boundary of the two tissues. These electronmicrographs illustrate again the close proximity of the two cuticles, as parts of each may be viewed in one thin section. The "Dalmatian-dog" pattern is smaller in figures from preserved specimens than in Figures 3 and 4 from fresh yellow cuticle.

SUMMARY

Electronmicrographs of the grasshopper egg membranes by the methods used show that:

A. There is no clearly resolvable internal structure of the chorion.
B. The yellow cuticle has no clearly resolvable internal structure, but has varying thicknesses due to minute ridges or projections on its outer surface.
C. The white cuticle is fibrous in structure.

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